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OXIDIZED LOW-DENSITY LIPOPROTEIN (LDL) FRACTIONS, CORRESPONDING ANTIBODIES, METHOD FOR OBTAINING SAME AND DIAGNOSTIC OR THERAPEUTIC USE

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(54) **Title**: OXIDIZED LOW-DENSITY LIPOPROTEIN (LDL) FRACTIONS, CORRESPONDING ANTIBODIES, METHOD FOR OBTAINING SAME AND DIAGNOSTIC OR THERAPEUTIC USE

(54) **Title**: FRACTIONS DE LDL OXYDÉES, ANTICORPS CORRESPONDANTS, PROCÉDÉ D'OBTENTION ET UTILISATION A BUT DIAGNOSTIQUE OU THÉRAPEUTIQUE

(57) **Abstract**: The invention concerns the use of myeloperoxidases for obtaining oxidized lipoproteins and corresponding monoclonal antibodies. Various fractions of oxidized LDL are isolated and new monoclonal antibodies are obtained from said fractions. Said antibodies are suitable for diagnostic, preventive and therapeutic use. Several productive lines of said antibodies have also been isolated and characterized. The invention also concerns tests and related kits for diagnosing and determining cardiovascular risk.

(57) **Abrégé**: L'invention concerne l'utilisation de myeloperoxidases pour l'obtention de lipoprotéines oxydées et des anticorps monoclonaux correspondants. Différentes fractions de LDL oxydées sont isolées et de nouveaux anticorps monoclonaux sont obtenus à partir de ces fractions. Ces anticorps sont aptes à être utilisés dans un but diagnostique, préventif ou thérapeutique. Plusieurs lignées productrices de ces anticorps ont également été isolées et caractérisées. L'invention propose aussi des tests et des kits associés pour le diagnostique et la détermination du risqué cardiovasculaire.

Fractions of Oxidized LDL, Corresponding Antibodies, Procedure for obtaining them, and Diagnostic and Therapeutic Uses.

This invention concerns the use of myeloperoxidase in obtaining oxidized lipoproteins and the corresponding monoclonal antibodies; the oxidized LDL fractions and the new monoclonal antibodies obtained from said fractions; the productive lines of said antibodies; the diagnostic and therapeutic uses of said antibodies and lines; and, in particular, a diagnostic test for determining cardiovascular risk in addition to diagnostic application kits.

Myeloperoxidase (MPO) is an enzyme present in neutrocytes and monocytes, helpful in catalyzing oxidation reactions in sub-endothelial space. It is well known that such oxidation uses hydrogen peroxide in the formation of hypochlorous acid, which is produced in the presence of MPO and chloride.

Moreover, low-density lipoproteins (LDL) in oxidized forms are known to be involved in certain cardiovascular pathologies—arteriolosclerosis in particular. And so, the presence of LDL, modified by hypochlorous acid, has been reported in atheromatous lesions.

The test, based on these lipoproteins and currently used to define a given patient's propensity for developing cardiovascular disease, makes use of a very powerful and non-physiological pro-oxidizing agent: copper sulphate ($CuSO_4$). However, this test is not particularly accurate and transpires under circumstances far removed from in-vivo conditions.

Many other diagnostic and analytical methods, based on LDL oxidation products, have become known. For example, see patent documents 5.874.313, WO 98/21581, WO 99/08109, WO 98/12561 and WO 98/10294. The last document proposes a diagnostic method based on measuring the 3-chlorotyrosine concentration in biological liquids and tissues since this substance is specifically produced by the MPO/Cl/H_2O_2 system. The document mentions that the measurement might be performed via immunoassay using antibodies produced against 3-chlorotyrosine.

Patent document WO 98/59248 (PCT/BE98/59248)2 reveals an immuno-detection procedure for LDLs, oxidized and modified by malondialdehyde (MDA) and associated antibodies. The oxidation, however, fails to simulate the natural process in an optimal manner.

One of the goals of this invention is to propose a diagnostic test capable of measuring parameters that enable earlier and more accurate detection of at-risk patients, with reference to an oxidative process for LDLs occurring in vivo.

The development of this test produced myeloperoxidase. More particularly, a human recombinant myeloperoxidase can be used with properties identical to those in natural myeloperoxidase.

This invention also proposes monoclonal antibodies, as well as the corresponding hybridomas, capable of recognizing specific fractions of lipoproteins oxidized via hypochlorous acid—for example, in the presence of myeloperoxidase—and thus capable of being used in immunodetection and the dosage of these oxidized lipoproteins. Such dosages may favorably reflect a factor for cardiovascular risk.

According to one aspect of the invention, we are proposing, generally speaking, a test for determining a patient's cardiovascular risk, including the following steps to be clarified later:

- anchoring an anti-fraction B monoclonal antibody onto a plate;
- extracting the oxidized LDLs from plasma through adsorption onto the monoclonal antibody;
- recognizing the anchored LDLs using an anti-apoB polyclonal antibody;
- revelation, via a marker, which may be, for example, a peroxidase coupled with the polyclonal antibody or a detection antibody: e.g., if the anti-apoB polyclonal antibody is a rabbit antibody, a goat anti-rabbit IgG (Fc) antibody coupled with alkaline phosphatase;
- determining the oxLDL concentration.

According to another aspect of this invention, it was noted that the oxidation speed of a given patient's LDLs constituted a promising method for defining said given patient's propensity for developing cardiovascular disease. As already mentioned, one of the main factors arising at an early stage in the formation of an atheromatous lesion is the peroxidation of the LDLs. Since the oxidized LDLs (oxLDL) are very rapidly trapped by the macrophages in the arterial wall, the mere measurement of their concentration could not be representative of their production in vivo. However, the likelihood of a given patient's LDLs undergoing peroxidative damage can be assessed using an in-vitro test.

According to still another aspect of the invention, we are proposing a test based on detection of natural auto-antibodies in plasma, directed against LDLs naturally oxidized via myeloperoxidase.

According to still another aspect of the invention, we are proposing an immunotherapeutic treatment method, which consists in treating a patient's blood to remove the oxidized LDL antigens from it. This method consists in running a patient's blood, or blood fraction, into a system in which it enters into contact with anti-fraction B antibodies, immobilized by an oxidized LDL fraction B—for example, on an immunoadsorption column—before being returned to the patient, with the blood or blood fraction at least partially freed of said oxidized LDLs.

DETAILED DESCRIPTION OF THE INVENTION

The invention is described in greater detail with reference to particular performance schemes described below and to the figures, which are appended as a non-exhaustive list of examples.

In the appendices,

- Fig. 1 is a chromatogram of oxidized LDL as described in the invention, showing the separation of the B, C and D sub-fractions;
- Fig. 2 represents a graphic of the sub-fraction concentration in terms of oxidation time;
- Fig. 3 illustrates the recognition of sub-fraction B via 3 monoclonal antibodies as described in the invention;
- Figures 4a through 4c illustrate the specificity of the antibodies vis-à-vis known epitopes, using the ELISA competition test;
- Fig. 5 illustrates the results of the ELISA test sandwich for measurement of circulating oxidized LDLs in 13 patients and 1 negative control;
- Fig. 6 is a diagram illustrating a test for measuring sensitivity to ex-vivo LDL oxidation;
- Fig. 7 illustrates the test results as described in Fig. 6 for 33 patients with respect to a control population.

One of the goals of the invention is to propose antibodies specifically directed against certain oxidized LDL sub-fractions as they are generated in biological systems. These oxidized LDL fractions may be prepared via oxidation with the H_2O_2/chloride system generating hypochlorous acid in the presence of myeloperoxidase.

Below, we shall describe methods for obtaining myeloperoxidase, for separating the fractions obtained, for producing hybridomas and antibodies specific to the oxidized fractions, as well as methods for using these antibodies in detecting and dosing oxidized LDL for diagnostic and therapeutic purposes.

1. Production of Myeloperoxidase

Myeloperoxidase can be produced from the CHO clone, which produces recombinant myeloperoxidase (rMPO). The clone, which was used as a part of this invention, is clone 24-1-7-57 and was grown in an Opticell culture (Charles River). In this system, the cells adhere to a ceramic medium (Opticore) with a 4,500 sq. cm. surface area and the culture medium is continuously infused. Several parameters are permanently controlled: the pH, the temperature, the oxygen content, the CO_2 content and the glucose concentration.

Every day, the lactate and glucose concentrations from the medium are measured as well as the peroxidase activity due to the myeloperoxidase synthesized and secreted into the medium.

The culture may be maintained for 2 months. Maximum production is about 30 days. Approximately every three days, the entirety of the culture medium is harvested, centrifuged and preserved.

Alternately, the cells may be kept in a cell factory culture with the culture supernatant also harvested every 3 days; the culture can be kept for 2 months.

Before every purification, it is necessary to purify the culture supernatants in order to eliminate cell debris. For that purpose, a filtration system, composed of a high-flow peristaltic pump (Watson Marlow 505 s) and a tangential filter (Ultrasette, Filtron), was used.

This instrumentation allows for simultaneous filtration and concentration of the culture supernatant prior to its deposit on the column. After a 10x concentration, the supernatant is diluted approximately 2 times until it attains a specific resistance of 4mS/cm, equivalent to that of the equilibriated column buffer. No loss of MPO was detected in the filtrate analyzed with a Western Blot; however, a loss of some 0.7% was revealed with the ELISA test.

This tangential filtration system therefore proves to be extremely useful and reliable in processing large volumes of culture supernatant, which is to be purified.

With each purification, some 10 liters of culture supernatant filtered, concentrated and equilibriated at the pH and ionic strength level are deposited on a Q Sepharose Fast Flow column (10cm x 20cm, 1570 ml of gel), equilibriated with a 20 mM pH 7.5 phosphate buffer. This anion exchange column, retaining contaminants—principally proteins, serumals—is directly connected to a CM-Sepharose column (3.5cm x 15cm), a cation exchanger on which the rMPO is kept and eluted using an NaCl gradient from 0 to 0.6 M (900 ml). The rMPO is unhooked from an approximately 0.28-M concentration of NaCl and exits in the form of a single spike protein. The peroxidase activity can be perfectly superimposed on a spike measured at 280 nm.

Analysis of the purity of the eluted fractions was carried out by electrophoresis on gel under non-reductive conditions and in the presence of SDS. With blue Coomassie staining, the NIPOR appears in the form of an 84-kDa band and a 94-kDa band. The second 94-kDa band is immunoreactive and corresponds to a minor MPO fraction.

The fractions were measured for protein concentration (Lowry assay) and peroxidase activity concentration (o-Dianisidine as a substrate). The most active fraction corresponds to the spike summit for elution and chlorination (with monchlorodimedon as a substrate).

According to the sugar analyses already performed, the 84-kDa and the 94-kDa bands correspond to forms of proMPO, revealing various degrees of glycosylation. The 84-kDa band would correspond, in large part, to the protein showing mannose-rich glycosylated chains, while the 94-kDa form would show chains of complex glycosylation all the way up to sialic acid.

From 80 liters of supernatant culture, 370 mg of pure MPO were obtained: i.e., 4.5 mg/l. The recombinant MPO appears in the form of 84-kDa and 94-kDa bands, having the same protein sequence though differing in the nature of their glycosylations.

2. Production of Oxidized LDL Sub-Fractions via Myeloperoxidase.

Oxidative changes, which may occur at the level of the LDLs, are largely of two types: on the one hand, the peroxidation of the lipids and, on the other, oxidative modifications at the level of the apoB. One of the most commonly used models for triggering oxidation of LDLs in vitro is based on the use of copper (Cu++) as an oxidizing agent (Esterbauer et al.). This model assesses, over time, the increase in the degradation products of the lipid components of the LDLs and provides information, for the most part, on the anti-oxidizing status of these lipoproteins. However, the use of Cu++ as a pro-oxidizing agent remains highly disputed since its involvement, at the level of cardiovascular pathologies, was not formally demonstrated. However, other oxidizing agents, such as hypochlorous acid, generated via MPO, seem to figure among the principal initiators of spumous cell formation and, thus, figure in the development of atheroma plaque. Indeed, MPO's involvement in in-vivo oxidation and in the pathogenesis of cardiovascular disease is strongly suggested by the presence of MPO and oxidized derivatives of tyrosine—especially dityrosine or, again, 3-chlorotyrosine—in atheromatous lesions. In fact, MPO uses hydrogen peroxide (H_2O_2) as a substrate to oxidize halides, such as chlorine ions, thus enabling the formation of hypochlorous acid—a highly toxic and microbicidal agent. It is important to note that, unlike the case for metallic ions, the oxidative debris produced by MPO involves proteins, for the most part—and, more particularly, apoprotein B in the cases of LDLs.

Conditions for LDL Oxidation

To better illustrate the aspect of the invention involving LDL changes produced by MPO, the hydrogen peroxide substrate was created in two different fashions: on the one hand, there was the progressive production of hydrogen peroxide as derived from the transformation of glucose via glucose-oxidase and, on the other hand, hydrogen peroxide was added directly into the oxidation medium.

The conditions for oxidation in MPO are as follows:

MPO/Glucose-oxidase system
278 mg LDL prot.
40 U MPO (peroxidase units with o-Dianisine as a substrate)
90 min. at 30°C

MPO/H_2O_2 system
1.6 mg LDL protein
8 U MPO
1 mM H_2O_2
5 min. at 37°C

Separation of the oxidized LDL sub-fractions can be performed by using Fast Protein Liquid Chromatography (FPLC) with a Mono Q anion-exchange column. The various

oxidation sub-fractions, indeed, show electronegative charges in relation to their degree of change. We observed that fractions (A, B, C, etc.) can thus be eluted from the column by using buffers containing NaCl-enhancing concentrations.

Following optimization of the conditions for elution, the coefficients of variation obtained for the various oxidized LDL fractions showed high reproducibility for the technique used (CV<5% for most spikes).

Fig. 1 illustrates the excellent separation of the oxidized fractions B, C and D using the lengthening of the saline plateaus corresponding to the migration of the fractions.

The FPLC results clearly show that the oxidative debris linked to MPO appears within the first 5 minutes whatever the oxidative system (glucose/glucose-oxidase or H_2O_2).

Fig. 2 illustrates the kinetics of appearance for oxidized fractions with hydrogen peroxide (MPO/ H_2O_2) for 179 µM H_2O_2 concentrations. We noticed that, after 5 minutes, the reaction is effectively over.

Characterization of the Oxidized LDL Sub-Fractions

Electrophoresis on Gel
Electrophoresis performed after oxidative stress shows that the debris produced by the MPO/Glucose-oxidase system consists largely in apoB protein fragmentations while the MPO/H_2O_2 system mainly produces aggregations.

In the MPO/Glucose-oxidase system, we observed a diminution in the polyunsaturated fatty acid proportions at the level of the phospholipids of fraction D with respect to the native fraction.

Change in the Fatty Acid Composition

	% in native fraction	fraction D
Arachidonic acid (C20: 4 n – 6)	8.2	6.3
Eicosapentaenoic acid (C20: 5 n – 3)	1.3	0.9
Docosahexanoic acid (C22: 6 n – 3)	4.6	3.1

The oxidized fractions obtained by the MPO/H_2O_2 system, however, show no significant modification in the fatty acid composition. The results indicate that the oxidation induced via glucose-oxidase produces peroxidative debris at the level of the LDL polyunsaturated fatty acids—which is not the case for the MPO/H_2O_2 case.

We see no differences in composition at the level of the cholesterol esters nor at the level of the triglycerides in the 2 oxidation systems.

We observed that the fractions issued from oxidation via the MPO/Glucose-oxidase system are the result of an MPO attack, followed by various radical reactions while the fractions deriving from oxidation via the MPO/H_2O_2 system seem to be entirely specific to an MPO attack.

The latter system will therefore be the preferred system in realizing the invention.

3. Production and Characterization of the Monoclonal Antibodies

The antigens against which the monoclonal antibodies of the inventions are directed are composed of different LDL oxidized fractions—more particularly, however, against fraction B.

As the patients' LDLs seem to oxidize more rapidly than those of normal subjects, one of the goals of the invention is to be able to differentiate between highly oxidized fractions (fractions C and D) and non-oxidized or slightly oxidized fractions (fractions A and B) in order to be able to determine the presence of fraction B quantitatively.

The invention also involves hybridomas that may be used in producing the above-mentioned antibodies.

The hybridomas are obtained, for example, in a traditional manner via fusion of non-secreting myeloma cells P3 x 3Ag8.653 (ATCC CRL-1580) with spleen cells from an immunized mouse, in the presence of polyethylene glycol 4,000.

The fused cells are distributed in pre-incubated boxes, containing 96 wells, with mouse peritoneal macrophages in the presence of a selection medium containing hypoxanthine, aminopterine and thymidine. The medium is replaced with a non-selective medium on day 7. Two weeks after fusion, the supernatants of the clones are screened for the presence of specific antibodies on each fraction A through D of the oxidized LDL, as well as on the MPO.

In these tests, the purified antigen (oxidized LDL fraction) is affixed to the plate, and the supernatant, alone or in competition with a purified fraction, is deposited on the plate. The anchored antibody is then revealed by a 2nd antibody directed against the mouse IgG and coupled with alkaline phosphatase.

We were therefore able to obtain two types of monoclonal antibodies, specifically recognizing LDLs altered by MPO. The one recognizes sub-fraction B, the other recognizes sub-fraction C and sub-fraction D.

In view of the characterization of the fractions obtained by the two oxidation systems (H_2O_2, glucose oxidase), the two antibodies recognize different epitopes.

Indeed, the anti-fraction B antibody would recognize an early change produced, for the most part, by HOCl while the anti-fraction C/D antibody would be directed against debris produced not only by HOCl but also by other radical rearrangements and/or reactions obtained via oxidation due to the MPO-glucose oxidase system. With the MPO/H_2O_2 system, we see no fraction D.

Production of Monoclonal Antibodies with Anti-Fraction B Specificity

A monoclonal antibody directed against a slightly oxidized epitope from LDLs (fraction B) was characterized. The mice were immunized using the same protocol as before but with this difference: the fraction B antigen was injected in the presence of aluminum hydroxide. This fraction B was oxidized using MPO in the presence of H_2O. Four monoclonal-antibody-secreting hybridomas—AG9, AE2, EB2 and EF2—directed against the fraction B of the LDLs were obtained.

The supernatants of the sub-clones—AE2, AG9, EB2 and EF2—were tested not only for the antigenic specificity against different fractions but also for their membership in various classes and sub-classes of immunoglobulins. These clones belong to the IgG class and IgGl sub-class; the light chain is type K. These hybridomas secrete monoclonal antibodies with specificity against fraction B oxidized via rMPO. Three secretor hybridomas were gradually adapted to the serum-free culture in a hybridoma-SFM medium (Life Technologies).

The supernatants for these 3 secretor hybridomas were purified by transition to a Protein A-Sepharose CL-4B, condition in PBS with a pH of 8. The elution was performed in 0.1 M citric acid at different pH levels (6.5 IgGl – 4.5 IgG2a – 3 IgG2b).

Production of Specific Monoclonal IgG's for Fraction B

Clone		total mg
AG948F4A2	Fraction 1	1.3 mg
	Fraction 2	2.6 mg
EB2E9G62IH2		4.53 mg
EB2G3G2		2.6 mg

The recognition of the fraction B antigen oxidized through the 3 monoclonal antibodies is very similar and is illustrated in Fig. 3 (antigen box).

The following table shows the characteristics for a several monoclonal antibodies, isolated within the framework of this invention by adopting the above-mentioned process.

Monoclonal antibodies	Specificity (oxidized LDL fractions)				typing	
	A	B	C	D	Heavy chain	Light chain
14A2G6	-	-	+	+	IgG1	K
10G1E11E9	-	-	+	+	IgG2a IgM	K
5C4B5E11	-	-	+	+	IgM	K
9B5D6E6	-	-	+	+	IgM	K
1E3E4C9	-	-	+	+	IgM	K
AG948F4A2	-	+	-	-	IgG1	K
EB2E9G621H2	-	+	-	-	IgG1	K
EB2G3G2	-	+	-	-	IgG1	K
3H5	+	+	+	-		
7F8	+	+	+	-		
8B1	+	+	-	-		

Hybridomas AG948F4A2, EB2E9G621H2, EB2G3G2 and 14A2G6 were filed with BCCM (Belgian Coordinated Collections of Microorganisms) on 19 December 2001 under the following provisional numbers:
1) 14A2G6 → LMBP 5828CB
2) AG948F4A2 → LMBP 5829CB
3) EB2E9G621H2 → LMBP 5830CB
4) EB2G3G2 → LMBP 5831CB

Characterization of the Monoclonal Antibodies

Specificity of the Epitope

Competitive ELISA tests were conducted in order to show the identity or the difference of the recognized epitopes via antibodies as described in the invention.

These tests were conducted on a plate covered with oxLDL. A marked antibody is added, placed in competition with growing concentrations of the other unmarked antibodies. A diminution in the signal indicates a competition for the same epitope. However, if the marked antibody recognizes an epitope different from that of the competitor, there will be no inhibition of the signal.

The marking of the antibodies with iodine-125 was performed using Pierce IODO-Beads, chloromine-T carriers. These marked antibodies were purified on 5-ml resin Fine Sephadex-G25 columns (Pharmacia) and characterized by TCA precipitation.

Figures 4a, 4b and 4c represent graphs for the ELISA competition tests between a marked antibody (AG948F4A2, EB2G3G2, 14A2G6) and the other unmarked monoclonal antibodies.

Given the inhibition of the signal, it may be concluded that the three antibodies directed against the fraction B of the oxidized LDLs, although showing different behavior, recognizes at least one part of the same epitope. However, antibody 14A2G6 recognizes an epitope different from the other antibodies with its signal remaining constant in the presence of the other antibodies.

Affinity of the Antibodies

The affinity of several antibodies, as described in the invention, was assessed by conducting competitive ELISA tests as mentioned above; the only difference was the placing of the marked antibody into competition with its cold homologue. We observed that the antibodies have an affinity, on the order of $5x10^{-8}$ M, for EB2E9G62IH2, EB22G3G2 [sic] and AG948F4A2, and, on a order of approximately 10^{-9} M, for 14A2G6. These values were measured by taking the antibody concentration, which induces a percentage of inhibition equal to 50%.

Specificity of the Recognition of Monoclonal Antibodies

We demonstrated that the four above-mentioned monoclonal antibodies are specific to the oxidized LDLs via the myeloperoxidase system. ELISA tests were conducted with fresh LDLs and 2-day-old LDLs; LDLs in the presence of HOCl and of LDLs oxidized via H_2O_2 (1mM and 2mM)—each time in the absence of rMPO; as well as with native and oxidized ApoB100, HDls, native Apo A-I or Apo A-I oxidized via MPO, and native VLDLs and oxidized VLDLs. Only oxidation via HOCl resulted in products that could be recognized by these antibodies.

We noted, however, that antibody 14A9G6 is less specific and also recognizes oxidized VLDLs via myeloperoxidase.

It is known, moreover, that these antibodies no longer recognize the fraction B from LDLs oxidized in copper or in MDA.

We further observed an equivalence in recognition of oxLDL and oxApoB100—which demonstrates that the oxidation of LDLs via MPO, under the described conditions, causes the oxidation of the protein part of the LDLs. Moreover, this equivalence allows us to use the oxApoB100, in a favorable manner, as a standard in the diagnostic tests producing the antibodies as described in the invention.

We also demonstrated that the antibodies, as described in the invention, are very well preserved for at least two years at 4°C, -20°C and -80°C (in the presence of glycerol for -20°C). These antibodies will be preserved preferably at approximately -20°C or -80°C.

4. ELISA Test for naturally Oxidized LDLs circulating in Plasma

ELISA tests measuring LDLs oxidized via MPO in circulation were thus developed as a part of this invention.

These tests, which do not use ex-vivo oxidation of LDLs (constituting a very delicate phase), can be conducted routinely.

One of the tests, as described in the implementation of the invention, can be summarized as follows:

- anchoring an anti-fraction B monoclonal antibody onto a plate;
- extracting the oxidized LDLs from the plasma through adsorption onto the monoclonal antibody;
- recognizing the anchored LDLs using an anti-apoB polyclonal antibody coupled with peroxidase;
- revelation through a mixture of H_2O_2/ortho-phenylenediamine (reading optical density at 490 nm)
- determining the oxLDL concentration in the sample, using a standard range for the fraction B of LDLs oxidized via MPO and isolated through FPLC (fraction box) or using an oxidized ApoB100 range.

To confirm the feasibility of the test as described in the invention, a standard for the oxidized LDL fraction box was applied to concentrations growing from 0 to 24 µg of protein per well). The 3 monoclonal antibodies (EB2E9G621H2, EB2-g3g2 and AG948F4A2), which recognize the oxidized fraction B, were tested.

The results show a good response for the three antibodies with respect to growing standard concentrations—linear in an initial phase, attaining a ceiling for the highest concentrations. This plateau can be explained by the upper detection limit of the test and/or by a disturbance due to the presence of NaCl in the fraction box (greater when the concentration in the box increases). Each curve was reproduced several times with the same fraction box and shows good reproducibility.

However, fraction boxes deriving from two different oxidations do not generate identical standard curves. Indeed, separation via FPLC allows for separation on the basis of the electrical charge, but does not provide information on the chemical nature of the epitope.

In order to obtain a pure standard for fraction box analyses—a standard recognized specifically by the monoclonal antibodies as described in the invention—we propose using de-lipidated oxLDLs—which corresponds to the purified oxApoB100 from LDLs oxidized via MPO as described in the following protocol (described by Socorro and Camero, J. Lipid Research: 20, 631, 1979).

The oxLDLs, as described in the invention, placed in a 50 mM Tris/HCL buffer solution with a pH of 8.8, are deposited onto a DEAE-Sepharose CL-6B (Pharmacia) column (1.5cm x 15cm) and a linear gradient of Triton X-100 from 0 to 2% is immediately applied (60ml). Following elution of the lipids with detergent, the LDL apo (oxApoB100) is eluted via a 50 mM Tris/HCL buffer with a pH of 7.4, containing 1M of NaCl.

The oxApoB100, analyzed by electrophoresis on a polyacrylamide gel, shows a single immunoreactive band with anti-apoB100 antiserum and monoclonal antibodies.

Moreover, preservation of the fractions can be favorably carried out in the presence of glycerol or sucrose.

The dose-response curve for native LDLs (with a plasma concentration) was also tested concurrently with the corresponding original plasma. As expected, growing concentrations of native LDLs produce a proportional increase in the signal. However, the response obtained for the plasma is null and clearly inferior to that of the native LDLs. This indicates that one or more components of the plasma can inhibit recognition of the LDLs oxidized via the monoclonal antibodies.

Among the plasma components likely to influence the link between the oxLDL and the monoclonal antibody, two large categories can be distinguished:

- lipoproteins, and especially particles containing apoB (VLDL and native LDLs); and
- proteins.

We confirmed the reliability of the test by verifying that the apoB not modified by MPO, and present both on the LDLs and the VLDLs, could not enter into competition with the modified apoB at the level of the monoclonal antibody.

The dose-response curve for the native LDLs shows a signal, which might reflect the presence of a small proportion of oxidized LDLs in these native LDLs.

We also verified that albumin does not interfere with the antibody-antigen link.

Several solutions are proposed for eliminating or minimizing the interference observed in the plasma.

First, the sample can be pre-purified by using semi-permeable membranes that enable elimination of molecules up to 100,000 Da. On the other hand, the IgG's can be removed using immuno-precipitation techniques.

We observed, however, that the interference can be limited by diluting the sample. In fact, an increase in interference was observed for weak plasma dilutions while greater dilutions tended to limit these disturbances. On the other hand, concurrently with sample dilution, various known techniques allow you to amplify the signal.

As an example, a preferred variant for the direct measurement test of circulating naturally-oxidized LDLs consists in an ELISA sandwich test as described below:

- coating of plates F 96 maxisorb nunc immuno plate no. 4 4 2 3 0 4 with the monoclonal antibodies (100 µl/well)

plate no. 1	Mab AG 948 F4 A2 (anti B) at 5.3 ng/well
plate no. 2	Mab EB 2 A 9 G 6 21 H2 (anti B) at 5.3 ng/well
plate no. 3	Mab EB2 G3 G2 (anti B) at 5.3 ng/well

in the 50 mM carbonate/bicarbonate buffer with a pH of 9.6

- saturation of the plates with 0.1% casein after washing in TBS Tween, 60 min, at 37°C;
- arrangement of the samples: plasma diluted 10 times in PBS pH 7.5 (H_2O HPLC), 60 min. at 37°C, then wash with TBS Tween. Ideally, a negative control and an oxidized B standard are also arranged on the plate;
- anchoring of 2nd antibody: apolipoprotein B 100 rabbit polyclonal antibody at 1 µg/ml, 60 min. at 37°C;
- anchoring of the coupled antibody: a goat anti-rabbit IgG (Fc) antibody coupled with alkaline phosphatase (Promega, cat. No. S3731), diluted 7,500 times in the casein buffer;
- revelation: using an alkaline phosphatase substrate: para-nitrophenyl phosphate at 1 mg/ml in the diethanolamine buffer pH 9.8.

We observe that the 3 antibodies provide comparable results.

An example of the results is taken up in Fig. 5 for 13 patients and a negative control (50 min. of revelation) by using the EB 2 A 9 G 6 21 H2 antibody already mentioned.

When reproducing the test with plasma samples diluted 50 times, the results are identical.

5. ELISA-Type Test for Measuring Sensitivity to Ex-vivo Oxidation of LDLs

As described in the invention, the kinetic characteristics of the oxidation of the LDLs via myeloperoxidase are exploited, with equal favor, for diagnostic purposes.

We thus carried out oxidation of LDL on a microplate in order to develop one of the tests, as described in the invention, based on the kinetics of oxidation.

This test involves 3 steps:

1) anchoring of the plasma LDLs onto an anti-apo B polyclonal antibody;
2) oxidation of the LDLs via the MPO/hydrogen peroxide system. 12.5 mU of MPO were used in the presence of 40 µM H_2O_2 per well; these conditions were then modified to slow down the oxidation by reducing the quantity of pro-oxidizing agents by 5 times and by using 2.5 mU of MPO and 80 µM H_2O_2 per well;
3) recognition of an epitope specific to LDLs oxidized via a monoclonal antibody as described in the invention.
4) recognition of the monoclonal antibody by IgG's coupled with alkaline phosphatase, anti-mouse IgG.

In order to optimize the oxidation conditions discriminating between patients and healthy individuals, we used the two obtained monoclonal antibodies (anti-fraction B and anti-fraction C/D) that were available.

The dosage procedure is illustrated in Fig. 6.

Several oxidation series were carried out on plasmas coming from both patients and healthy volunteers. Oxidation of the LDLs was followed, over time, by the appearance of epitopes B and C. It soon seemed necessary to establish a threshold oxidation value, defining normal or pathological oxidation. To that end, the measurement of a C/B ratio can constitute an internal standard for the reaction.

Twenty-five samples coming from healthy volunteers were tested in order to determine an average "normal" value for the C/B ratio; the average obtained reflects a C/B ratio of 1.26 with a standard deviation of 0.16. The established "normal" range thus reflects a C/B ratio, going from 0.94 to 1.58 (average $\pm$ 2 SD).

Plasmas from 33 patients were tested and were compared with the "normal" range established over plasmas from healthy individuals. Eleven of them differed from the "normal" (Fig. 6). Among the patients showing sensitivity to oxidation greater than the "normal," it was particularly interesting to note that some showed no classic risk factors such as elevated triglycerides and/or cholesterol, or more recent factors like small, dense LDLs or Lp(a), and yet presented severe coronary artery disease (unstable angina, infarct, etc.)

This test therefore showed itself to be of considerable interest in the area of primary prevention by highlighting a potential risk while the other parameters in current use proved to be negative.

Fig. 7 illustrates a comparison of LDL sensitivity to oxidation for 33 patients with respect to a control population; the results are expressed in percentages with respect to the control.

Intra-assay reproducibility, tested via production of several kinetics on the same plate, is entirely acceptable. However, inter-assay reproducibility, tested via comparison of the results obtained for the same sample on different plates, is poor. A control sample will then be introduced on each plate so that we can interpret the results obtained on different plates.

The obtained kinetics shows that the results obtained with the anti-fraction B antibody are highly reproducible while those obtained with the anti C/D show variations. In fact, it was verified—by taking three blood samples from the same volunteer over a span of several days—that the same individual taken at different times gave comparable results.

It may thus be concluded that the measurement of the kinetics of LDL oxidation can be performed under good conditions by using the anti-fraction B antibody.

6. ELISA Test for measuring Circulating Auto-Antibodies directed against Oxidized LDLs (fraction B).

According to still another aspect of the invention, we propose a test based on the detection of natural antibodies (designated hereinafter as auto-antibodies) in plasma, directed against LDLs oxidized via myeloperoxidase.

In fact, we observed that plasma contains auto-antibodies directed specifically against fractions of LDLs oxidized via myeloperoxidase in the presence of H_2O_2, and not against the fractions obtained via oxidation with other agents such as copper sulfate, AAPH or azoinitiator.

The rate of auto-antibodies recognizing these oxidized LDLs is a reflection of the oxidative state in as much as the modification of the LDLs results in the appearance of new epitopes, which render the lipoproteins more antigenic. The dosage also makes it possible to assess the ability of the immune system to respond to the appearance of these modified LDLs.

As described in the invention, we are therefore proposing an ELISA-type test, which would make it possible to determine quantitatively the presence of auto-antibodies against specific sub-fractions in patients showing cardiovascular pathology.

One of the tests, as described in the implementation of the invention, can be summarized as follows:

- anchoring of oxidized LDLs or VLDLs oxidized via MPO to the plate;
- addition of 1/10 and 1/50 diluted sera;
- recognition of anchored antibodies using a goat anti-human IgG polyclonal antibody (heavy and light chain) coupled with alkaline phosphatase;
- revelation via the substrate of the alkaline phosphatase (paranitrophenylphosphate in a diethanolamine buffer);
- reading at 410 nm with reference to 630 nm.

The following table indicates the responses observed for 5 patients in the form of ratios between the signal obtained with LDLs oxidized in various manners and the signal from the corresponding blank: i.e., for the native LDLs. A ratio of 1 (or <1) signifies that the sample does not contain auto-antibodies directed against the oxidized LDLs. Conversely, we can postulate that a ratio of > 2 is the result of the presence of auto-antibodies.

Patient	LDLs oxidized via Cu	via AAPH	via MPO
1	1.37	1.34	3.27
2	<1	1.18	<1
3	<1	1.25	3.91
4	<1	1.47	3.74
5	1.04	1.22	<1

In 3 of the 5 subjects, we observe the presence of auto-antibodies directed against the LDLs oxidized via MPO while no subject appears to contain auto-antibodies against the LDLs oxidized via copper or AAPH.

The invention thus demonstrates the feasibility and the advantages of the dosage of LDLs modified by the action of the MPO in circulation.

7. Therapeutic and Preventive Applications

According to another aspect of the invention, we are proposing a method of immunotherapeutic treatment consisting in treating a patient's blood to remove the antigens from it (oxidized LDL fractions, especially fraction B). This method consists in running a patient's blood, or blood fraction, into a system in which it will enter into contact with anti-fraction B antibodies, immobilized by an oxidized LDL fraction B, for example, on a specific immunoadsorption column, before being returned to the patient. The blood or blood fraction is freed from said auto-antibodies.

According to still another aspect of the invention, we are proposing passive immunization through administration of antibodies, as described in the invention, to an at-risk patient via injection or perfusion. This type of treatment could be applied, for example, after a coronary bypass operation.

Finally, according to another aspect of the invention, we are proposing injecting a patient with oxApoB100 proteins capable of eliciting a favorable immune response.

CLAIMS

1. Composition comprised of at least one oxidized LDL fraction, obtained through in-vitro oxidation of LDL using hypochlorous acid or a biological or chemical system producing hypochlorous acid.

2. Composition as described in the preceding claim, characterized by the fact that the oxidation is carried out through the action of hydrogen peroxide and myeloperoxidase.

2. [sic] Composition comprised of at least one oxidized LDL fraction as can be obtained via hydrogen peroxide or the glucose/glucose-oxidase pair in the presence of myeloperoxidase and chloride ions.

4. Composition as described in claim 1, 2 or 3, purified via chromatography.

5. Composition as described in the preceding claim, obtained through Fast Protein Liquid Chromatography (FPLC) separation.

6. Composition as described in the preceding claim, obtained through salt-enhancing buffer concentrations.

7. Oxidized LDL B fraction as can be obtained through FPLC-type chromatography from oxidized LDL in the presence of myeloperoxidase as illustrated in Fig. 1.

8. Composition as described in any of the preceding claims, which were also purified via immunoaffinity.

9. Composition as described in the preceding claim, purified via immunoaffinity, using a column composed of a monoclonal antibody coupled with a matrix and directed against said fraction.

10. Composition as described in the preceding claim in which the column is an activated Sepharose column or a glass bead column.

11. Composition as described in any of the preceding claims, characterized by the fact that it constitutes, or is identical to, a (B) sub-fraction, the oxidized LDL sub-fraction closest to native (A) LDLs under Fast-Protein-Liquid-type Chromatography with a Mono Q anion-exchange column.

12. Composition as described in any claim 1 through 8, characterized by the fact that it is identical to, or constitutes, a (C) sub-fraction, the oxidized LDL sub-fraction which follows the (B) sub-fraction under Fast-Protein-Liquid-type Chromatography with a Mono Q anion-exchange column.

13. Composition comprised of an oxidized fraction of LDL, obtained through in-vitro oxidation of LDL via the glucose/glucose oxidase pair in the presence of myeloperoxidase, characterized by the fact that it includes the D sub-fraction, the oxidized LDL sub-fraction furthest away from native LDLs under Fast-Protein-Liquid-type Chromatography with a Mono Q anion-exchange column.

14. Composition as described in claim 13, characterized by the fact that it is comprised of a mixture of C and D sub-fractions.

15. Composition as described in claim 1 or 2 in which the myeloperoxidase used is a human recombinant myeloperoxidase.

16. Monoclonal antibody directed against one or more components of a composition as described in any of claims 1 through 15.

17. Procedure for obtaining oxidized fractions of LDL, characterized by the fact that the LDLs are oxidized through the addition of hydrogen peroxide in the presence of myeloperoxidase and the resulting oxidized fraction is separated and then fractionated by Fast-Protein-Liquid-type Chromatography (FPLC), using salt-enhancing buffer concentrations.

18. Use of oxidation fractions as described in any of claims 1 through 15 or obtained according to the description in the preceding claim for obtaining monoclonal antibodies.

19. Antigen formed by an isolated oxidation fraction as described in the procedure mentioned in claim 14.

20. Antigen as described in the preceding claim, formed by the B fraction, the oxidized LDL fraction closest to native LDLs under Fast-Protein-Liquid-type Chromatography with a Mono Q anion-exchange column.

21. Antigen as described in claim 16, formed by the C fraction or the B fraction or by a mixture of these LDL oxidation fractions.

22. Monoclonal antibody directed against one of the antigens mentioned in claims 19 through 21.

23. Monoclonal antibody produced by hybridoma AG948F4A2, filed with the BCCM under no. LMB 5829CB on 19 December 2001.

24. Monoclonal antibody produced by hybridoma EB2G3G2, filed with the BCCM under no. LMBP 5831CB on 19 December 2001.

25. Monoclonal antibody produced by hybridoma EB2E9G621H2, filed with the BCCM under no. LMBP 5830CB on 19 December 2001.

26. Monoclonal antibody produced by hybridoma 14A2G6, filed with the BCCM under no. LMBP 5831CB on 19 December 2001.

27. Hybridoma AG948F4A2, filed with the BCCM under no. [no number given] on 19 December 2001.

28. Hybridoma EB2G3G2, filed with the BCCM under no. [no number given] on 19 December 2001.

29. Hybridoma EB2E9G621H2, filed with the BCCM under no. [no number given] on 19 December 2001.

30. Hybridoma 14A2G6, filed with the BCCM under no. [no number given] on 19 December 2001.

31. Use of an antibody as described in claim 16 or claims 22 through 26 for an ex-vivo LDL oxidation test for a patient.

32. Use of an antibody as described in claim 16 or claims 22 through 26 for a test to measure oxidized LDLs circulating in human blood.

33. Use of an oxidized LDL fraction, isolated as per standard, for determining oxidized anti-fraction auto-antibodies in a patient.

34. Use as described in claim 33 in which the oxidized fraction is the B fraction.

35. Test, especially for determining the cardio-vascular risk of a patient, characterized by the fact that it includes the following steps:
- anchoring a B anti-fraction monoclonal antibody onto a solid substrate;
- extracting the oxidized LDLs from the plasma through adsorption onto the monoclonal antibody;
- recognizing the anchored LDLs using an anti-apoB polyclonal antibody;
- performing the quantitative discovery of the polyclonal antibody;
- determining the oxLDL concentration.

36. Test as described in claim 24 in which the quantitative discovery is carried out via the action of alkaline phosphatase coupled with an antibody directed against the anti-apoB polyclonal antibody.

37. Test as described in the preceding claim in which the anti-apoB polyclonal antibody is a rabbit antibody and the antibody coupled with the alkaline phosphatase is a goat anti-rabbit IgG (Fc) antibody.

38. Test as described in claim 35 in which the anti-apoB polyclonal antibody is coupled with a peroxidase.

39. Test as described in claim 35 in which the oxidized LDL concentration in the sample is determined by comparison with a standard range or sample, taken either from the oxidized LDL B fraction through myeloperoxidase and isolated by FPLC (fraction box) or from ApoB100 oxidized via hypochlorous acid or a system producing hypochlorous acid.

40. Test as described in claim 35 or claim 39 in which the plasma is first diluted between 5 times and more than 40 times.

41. Test as described in any of claims 35 through 40 in which the plasma IgG's are eliminated through immunoprecipitation techniques.

42. Procedure for determining the circulating rate of oxidized LDL, characterized by the fact that it uses monoclonal antibodies, obtained from at least one fraction of the ex-vivo LDLs oxidized through myeloperoxidase.

43. Procedure for determining the rate of auto-antibodies directed against the oxidized LDLs, characterized by the fact that it uses fractions of oxidized LDLs as described in claim 16 or claims 22 through 26.

44. Procedure for determining the sensitivity to oxidation through myeloperoxidase of the LDLs present in the plasma, characterized by the fact that it uses monoclonal antibodies as described in claim 16 or claims 22 through 26.

45. Diagnostic procedure for the prevention of cardio-vascular accidents giving rise to at least one of the three procedures in claims 42 through 44.

46. Diagnostic procedure for assessing the severity of the cardio-vascular risk of a patient, characterized by the fact that it is based on the following three parameters:
- circulating rate of LDLs oxidized via myeloperoxidase;
- rate of antibodies directed against the LDLs oxidized via myeloperoxidase;
- sensitivity to oxidation through the myeloperoxidase of the LDLs present in the patient's plasma.

47. Pure standard for the B fraction oxidized through myeloperoxidase, characterized by the fact that it is obtained from said fraction via passage to an immunoaffinity column, including an anti-fraction B monoclonal antibody.

48. Oxidized ApoB100 protein as can be obtained and purified from an LDL oxidized via the action of HOCL.

49. Protein as described in the proceeding claim, purified by passage onto an immunoaffinity column, including an anti-fraction B monoclonal antibody.

50. Standard used in diagnostic tests based, at least in part, on the presence of oxidized LDLs, including a protein as described in any of the three preceding claims.

51. Diagnostic kit including at least one monoclonal antibody as described in claim 16 or claims 22 through 24.

52. Kit as described in claim 35, also including a standard or range of standards consisting of one or [missing text] of the compositions as described in claims 1 through 15 and 47 through 50.

53. Test, especially for determining the cardio-vascular risk of a patient, characterized by the fact that it includes the following steps:

- anchoring oxidized LDLs onto a solid substrate;
- anchoring an anti-fraction B monoclonal antibody to the aforesaid anchored oxidized LDL;
- associating the oxidized LDLs of the plasma with said antibody and liberating the monoclonal antibody of said substrate;
- determining the monoclonal antibody that remains anchored;
- determining the oxidized LDL concentration of the plasma.

54. Treatment procedure consisting in treating the blood or portions of the blood of a patient in order to remove the oxidized fraction components of the LDL with antibodies as described in claim 16 and claims 22 through 24, immobilized on a support such as an immunoadsorption column, before being returned to the patient.

54. [sic] Treatment procedure consisting in treating the blood or portions of the blood of a patient in order to remove the B fraction of the oxidized LDLs through contact with the anti-fraction B antibodies immobilized on an immunoadsorption column, before being returned to the patient.

55. Therapeutic or preventive treatment procedure consisting in administering antibodies, as described in claim 16 and claims 22 through 24, to a patient via injection or perfusion.

56. Therapeutic or preventive treatment procedure consisting in administering a protein, as described in claims 48 or 49, to a patient via injection or perfusion,.

Fig. 1 [no French]

Fig. 2
[running vertically up left side: Alre %]
[at bottom under graph: Duration of Oxidation (min.)]

Fig. 3
[at top, above graph: Recognition of antigen Box via 3 anti-box monoclonal antibodies]
[bottom right, below graph: µg of Box/well]

Fig. 4a
[at top, above graph: marked AG948F4A2]
[running vertically up left side: % cpm (1 min.)]
[at bottom, first line under graph: marked Ac]
[at bottom, second line under graph: unmarked inhibitors (M)]

Fig. 4b
[at top, above graph: marked EB2G3G2]
[running vertically up left side: % cpm (1 min.)]
[at bottom, first line under graph: marked Ac]
[at bottom, second line under graph: unmarked inhibitors (M)]

Fig. 4c
[at top, above graph: marked 14A2G6]
[running vertically up left side: % cpm (1 min.)]
[at bottom, first line under graph: marked Ac]
[at bottom, second line under graph: unmarked inhibitors (M)]

Fig. 5
[at bottom under graph: plate no. 3 EB2E9G621H2 50 min. of rev.]

Fig. 6

[figure] washing Coating	[figure] washing Inc. plasma	[figure] Oxidation
[figure]	[figure]	[downward-pointing arrow] washing
[figure] washing Revelation	[figure] washing Conjugated Incub.	[figure] Monoclonal Incub.
[figure] native LDL	[figure] oxidized LDL	[figure] GaM-AP
[figure] Poly. Lap. Anti native LDL		[figure] MAb. Anti oxidized LDL (c,d) or b

Fig. 6
[at bottom left, under graph: "normal" range]
[at bottom right, under graph: subject exiting "normal" range]